Naval Medical Research Inst

STUDIES ON THE FREEZING RESISTANCE OF INTERTIDAL MOLLUSCS

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This paper will report a series of freezing studies on two common mollusc species inhabiting the temperature and subarctic coast line. The mussel, Mytilus edulis, which spends the winter periodically exposed to severe temperatures, shows an ability to survive freezing at a body temperature of -10°C for extended periods. The hardshell clam, Venus mercenaria, is a mud or sand burrowing animal protected from exposure to air, and can survive freezing only to -6°C. We became interested in these species as a result of reports that Mytilus could withstand months of freezing to -20°C. Our own field and laboratory experiments cast serious doubt on these observations. Our work showed that both Mytilus and Venus can survive exposure to lower ambient temperatures for short periods, but that this represents the time required to remove sufficient heat from these large animals to lower the body temperature below the value which the tissue can tolerate. That is, a Mytilus 7cm long can survive -20°C for two hours, the time required to lower its body temperature to below the critical -10°C as seen in Figure 1. Before the tide goes out, Mytilus fills its mantle cavity with sea water and closes its shell. This adds considerably to its heat capacity and can be held as a major factor in the ability of the mussel to withstand the ravages of winter. By the time its body temperature has dropped significantly, the tide has returned to protect it.

This may explain the evolutionary development of the resistance, but resolves no problems on the mechanism. To this end I would like now to report two sets of studies on isolated tissues: Morphological changes in frozen tissue seen with the electron microscope, and studies of the time-temperature relationships of injury from tissue freezing, with and without cryoprotective agents.

We have made a preliminary survey of the results of freezing on fine structure using the electron microscope. Since we have an organism which can survive a rather severe freezing, it should be possible to divide any structural changes in tissue into those attendant upon freezing only, and those which occur in conjunction with tissue death. So far, we have compared only the structure of Mytilus tissue frozen nonlethally with that frozen lethally.

Figure 2 shows tissue frozen non-lethally to -10° C and thawed in fixative. No abnormalities are evident in cilia, microvilli, cell membranes, junctional regions or mitochondria as compared to unfrozen tissue. Recall that this tissue underwent an osmotic change almost as great as that in Figure 3 which shows the effects of sample, the mitochondria are bloated, cristae are distorted, cell membranes destroyed and the microvilli are distended.

I want next to report on studies of the survival of excised pieces of gill tissue following exposure to varying subfreezing temperatures. Small strips of gill tissue were placed in artificial sea water medium into 7x100mm tubular freeze-drying ampules, and frozen in an alcohol

bath. Freezing was initiated at a relatively high temperature, usually above -3° C. In the small vials used, temperature equilibrium could be achieved in seconds, rather than the better part of an hour as required for whole animals. Exposure time of a given temperature could therefore be equated for all practial purposes to total exposure. All tissue was thawed by placing the tube directly into room temperature water. A semi-quantitative estimate of the tissue vitality was made by determining the percentage of cells beating plus a figure of merit reflecting the speed and quality of the beat.

The results of these studies are summarized in Figure 4. The ordinate is the time required for cell viability to drop to 40% of its initial value. The tissue survives indefinitely at temperatures above its critical temperature, but below this, survival falls off precipitously to some small but finite value at lower temperatures. The critical temperature for these excised tissues seems slightly higher than that for intact animals, perhaps because some component of the repair mechanism is absent.

In our studies on whole animal survival we had made calorimetric determinations of the amount of water frozen out at various temperatures. The results may be seen in Figure 5. Note first that in both of these species, an equal amount of dehydration has occurred at the critical temperature. This aspect is fully discussed in Dr. Meryman's presentation.

(Sapporo presentation: enclosed) But of importance here is the fact that much less water freezes at a given temperature in Mytilus, despite the fact that there is more water present (88%, compared to 82%.) This implies the presence of a small molecule capable of binding large amounts of water: i.e., a natural cryoprotective agent.

Two approaches toward the identifying of this agent were used: chromatographic separation of tissue extracts, and a series of survival tests on tissue following administration of known cryoprotective agents.

The chromatography has so far proved singularly unproductive. We had assumed that the cryoprotective agent would be a polyhydric alcohol, to which the cell membranes were freely permeable. We used the procedure described by Salt for the detection of glycerol in insects. This procedure, incidentally gave an intense reaction with DMSO.

Published work on Mytilus reports negative findings for tests of both DMSO and Glycerol. We found some glycerol in this species, perhaps half a percent, and hardly enough to consider significant.

The procedure also revealed a chloroform-soluble component, a concentration of the intracellular compound taurine, and another component, probably mucopolysaceharide. In Venus, in which a cryoprotective agent was not expected, the same compounds were revealed, plus traces of two compounds not yet identified.

Of the cryoprotective agents added artificially to the specimens,

only DMSO has shown promise. It can protect Venus gill up to 24 hours at -30° C, in concentrations as low as 5%. Inositol proved more injurious than no additive at 5% concentration. Glycerol produced damage to tissues in concentrations greater than 10%. Nonetheless, it provided some protection, allowing tissue of both species to survive to -15° C.

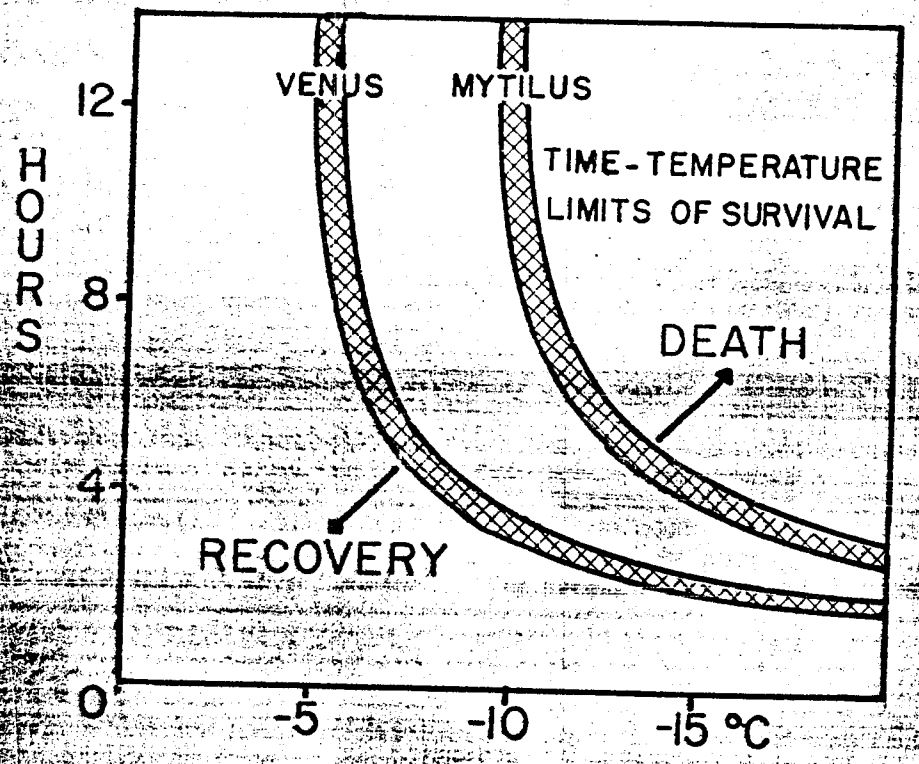
VENUS
MYTILUS
TIME-TEMPERATURE
LIMITS OF SURVIVAL
DEATH
RECOVERY
HOURS
12
8
4
0
-5
-10
-15 °C

Fig 2

Fig 3

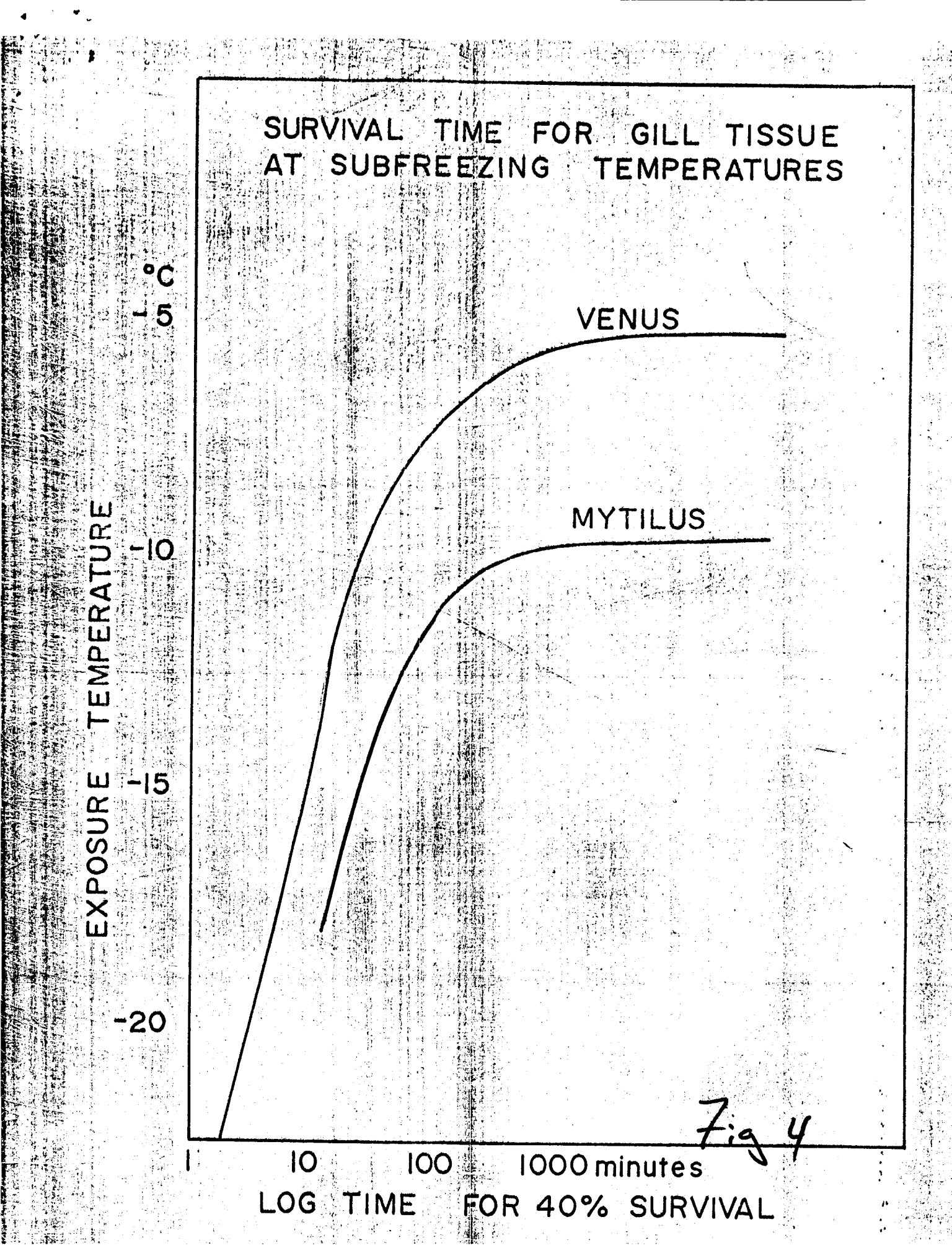
SURVIVAL TIME FOR GILL TISSUE
AT SUBFREEZING TEMPERATURES
VENUS
MYTILUS
EXPOSURE TEMPERATURE °C
-5
-10
-15
-20
1
10
100
1000 minutes
LOG TIME FOR 40% SURVIVAL

Fig 4

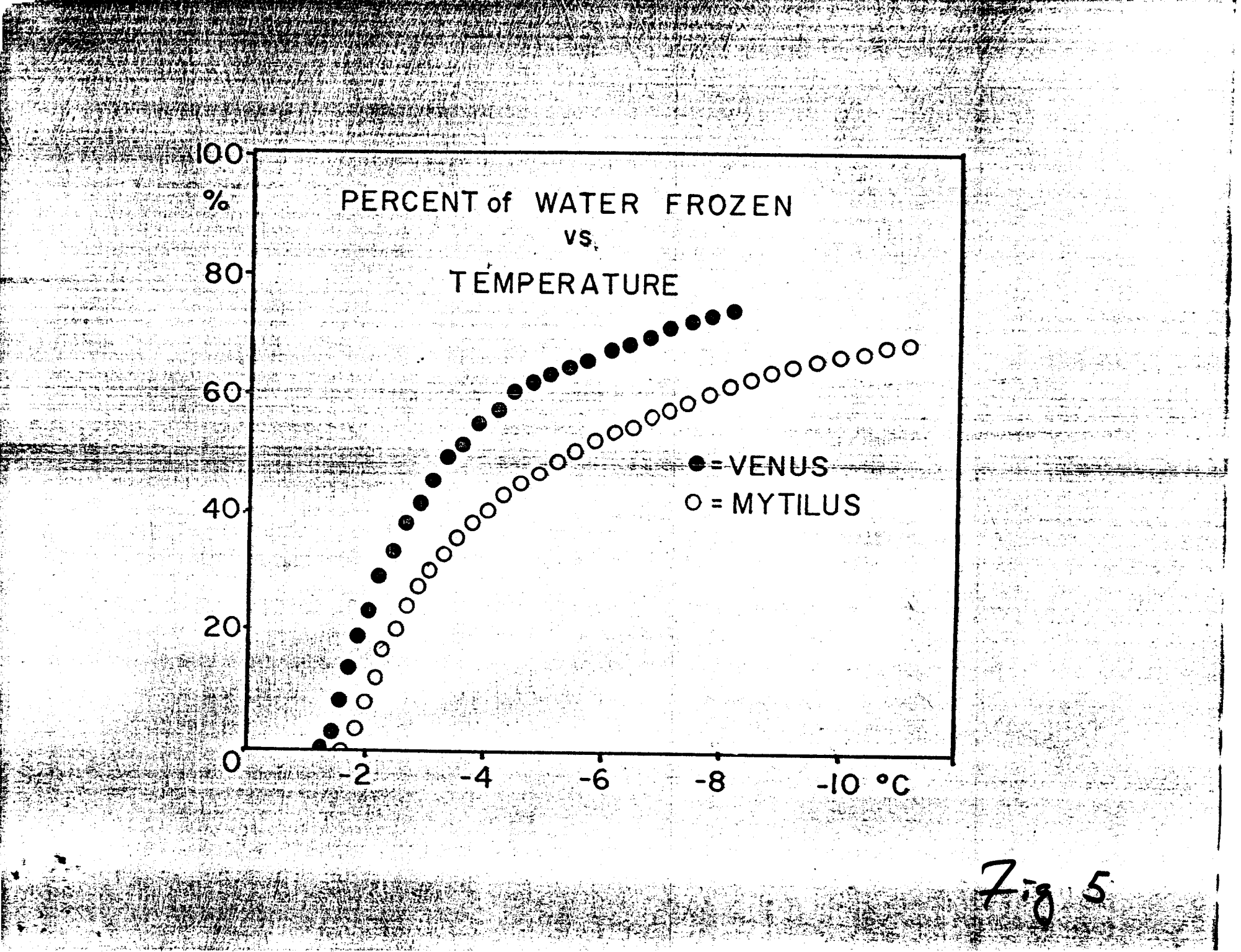
PERCENT of WATER FROZEN
vs.
TEMPERATURE
100
%
80
60
40
20
0
-2
-4
-6
-8
-10 °C
● = VENUS
○ = MYTILUS

Fig 5